

Investigation into the Alternatives to Acetonitrile for the Analysis of Peptides on a VariTide RPC

Application Note

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Introduction

Recently, investigative work has been carried out by Agilent Technologies to determine solvents that can be used instead of acetonitrile for reversed phase HPLC. There is currently a global shortage of acetonitrile, therefore many laboratories are looking to minimize their day-to-day use of this solvent.

Iso-propanol and n-propanol have been identified as two solvents that can be used for this purpose, and give good selectivity for peptides (as shown in Application Note 5990-7740EN). This report discusses their use with a VariTide RPC column, which is specially developed for peptide purification applications.



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Materials and Methods

Sample Preparation

A mixture of the following 4 peptides was made up to contain 1 mg/mL of each in a solution of 0.1% TFA in water:

Oxytocin	MW: 1007
Angiotensin II	MW: 2046
Angiotensin I	MW: 1296
Insulin	MW: 5808

Results

With acetonitrile, there is very little background absorbance from the changing composition of the eluent throughout the gradient, therefore the baseline remains relatively stable for the duration of the run.

Two different solvents were replaced for acetonitrile, and the peptide mixture injected in each case. Due to the differences in the organic strength of each solvent, the start point and end point of the actual gradient profile needed to be modified to elute the peptides during the run.

This data was then used to calculate the percentage organic required to elute each peptide with each alternative solvent, which is summarized in the following chart (Figure 1):

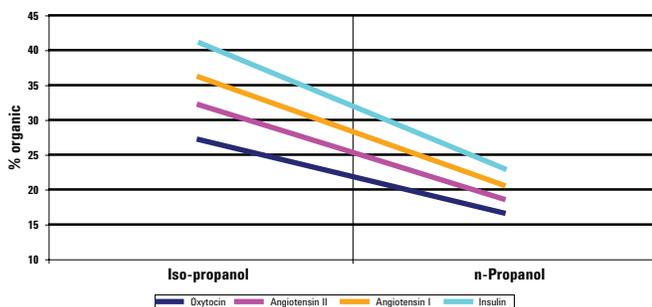


Figure 1. Peptide retention as a function of organic type and content.

Iso-propanol

Iso-propanol (also known as propan-2-ol, or IPA) is more commonly used as a modifier in normal phase chromatography, however it may also be used for reversed phase HPLC due to its miscibility with a large range of different solvents. Iso-propanol has 6 times the viscosity of acetonitrile and gave very high back pressure on our HPLC system. Increasing the temperature reduces the viscosity and operating pressure, and increases the efficiency of the separation.

The mobile phase conditions required to generate the data for the plot in Figure 2 are shown below:

Eluent A:	0.1% TFA in 1% iso-propanol: 99% water
Eluent B:	0.1% TFA in 99% iso-propanol: 1% water
Gradient:	0 – 50% B in 15 minutes
Flow Rate:	0.5 mL/min
Temperature:	40 °C
Injection Volume:	20 µL
Detection:	UV at 220 nm

A very good separation of the peptides can be obtained with IPA, as can be seen in Figure 1. All four peptides elute within a wide range of % organic values (27 – 41%), and are therefore well resolved.

IPA gives maximum UV absorbance at 204 nm, therefore under these conditions a high background absorbance is obtained during the course of the gradient resulting in a noisy and drifting baseline.

n-Propanol

n-Propanol (also known as propan-1-ol) is a primary alcohol like methanol and ethanol, therefore can also be used for reversed phase HPLC analysis.

As an isomer of iso-propanol, it has very similar physical properties but is slightly less viscous. It also needs to be run at 40 °C to keep back pressure down and improve overall column efficiency. The gradient profile was modified as follows to determine the elution profile for all four peptides:

Eluent A:	0.1% TFA in 1% n-propanol: 99% water
Eluent B:	0.1% TFA in 99% n-propanol: 1% water
Gradient:	0 – 50% B in 20 minutes
Flow Rate:	0.5 mL/min
Temperature:	40 °C
Injection Volume:	20 µL
Detection:	UV at 220 nm

Table 1. Comparison of column efficiency and peptide resolution for all solvents.

Solvent	Efficiency				Resolution (1,2)	Resolution (2,3)	Resolution (3,4)
	Oxytocin (1)	Angiotensin II (2)	Angiotensin I (2)	Insulin (4)			
Iso-propanol	171,300	187,700	94,700	125,200	4.69	2.61	2.04
n-Propanol	102,200	120,800	120,800	190,600	2.84	2.98	4.08

n-Propanol is a stronger solvent than iso-propanol, therefore much less is required in the mobile phase to elute the peptides. However, this solvent also gives baseline drift due to its absorbance at 220 nm.

Table 1 summarizes the differences in the selectivity of the 2 different mobile phase solvents, in terms of the column efficiency and resolution between pairs of peptides (under their respective gradient profiles).

Conclusion

These results show two different solvents that can be used as alternatives to acetonitrile for the reversed phase HPLC analysis of peptides.

With a VariTide RPC column, either iso-propanol or n-propanol could be used, however, the best separation of the compounds in our test sample was with n-propanol. The gradient profile used gives a faster separation between the 4 peptides, with very good resolution between each one (as shown in Figure 2)

However, these solvents require elevated temperatures to reduce the viscosity and back pressure, resulting in an increase in the efficiency.

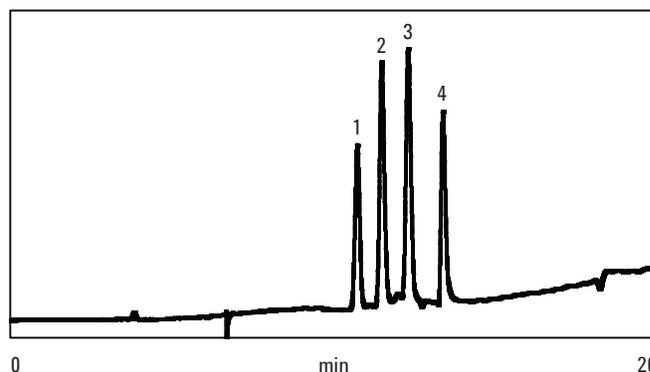


Figure 2. Peptide mixture on VariTide RPC 250 x 4.6 mm ID column at 0.5 mL/min, 40 °C. Gradient elution of 0-50% B in 20 minutes. Compounds: 1. Oxytocin, 2. Angiotensin II, 3. Angiotensin I, 4. Insulin.

These solvents do introduce a certain degree of background absorbance when run with a UV detector at 220 nm, resulting in baseline drift or noise, therefore an alternative detector such as an Agilent 385-LC ELSD could be considered which would easily evaporate both solvents and give flat stable baselines.

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Published in UK, May 17, 2011

5990-8145EN



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